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The Pharmacodynamic Functions of Low-Dose Rapamycin as a Model for Universal Influenza Protection

William Gust
University of Mississippi. Sally McDonnell Barksdale Honors College

THE PHARMACODYNAMIC FUNCTIONS OF LOW-DOSE RAPAMYCIN AS A MODEL FOR UNIVERSAL INFLUENZA PROTECTION

By:
William Gust

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Approved by

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Advisor: Dr. John M. Rimoldi

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Reader: Dr. Joshua Sharp

_____
Reader: Professor James Cizdziel

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ABSTRACT
WILLIAM GUST: The Pharmacodynamic Functions of Low-Dose Rapamycin as a Model for Universal Influenza Protection
(Under the Direction of Dr. John M. Rimoldi)

One of the most sought after breakthroughs in modern influenza research is the creation of a universal influenza vaccine that would protect against all strains of avian influenza. An unlikely model for this kind of protection might be found in the mTOR inhibitor rapamycin. Traditionally used to prevent organ transplant rejection, low doses of rapamycin have been found to protect mice against infection with distinct subtypes of influenza. However, it is not understood how a partial block in this pathway provides optimal heterosubtypic immunity. Interestingly, previous experiments have shown that low dose rapamycin inhibits germinal center formation to reduce antibody class switching and generate cross-protective influenza antibodies. Yet, it is not known how these antibodies, which do not neutralize the virus, enhance protection against distinct influenza subtypes.

The focus of my research was to further characterize the antibody protection and mTORC1 downstream effector regulation mediated by low dose rapamycin. Significant mechanisms of viral clearance by non-neutralizing antibodies include complement-mediated phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC). To investigate whether these mechanisms are required for rapamycin-mediated antibody protection *in vivo,* we inhibited the complement system *in vivo* using Cobra Venom Factor (CVF), depleted natural killer cells *in vivo* using an anti-NK1.1 antibody, and compared the efficacy of rapamycin treatment between control and depleted mice following H5N1 infection in both cases. We also performed an *in vitro* ADCC assay with serum from PBS or rapamycin-treated mice. Based on our data, we concluded that

rapamycin-mediated antibodies do not use the complement system or ADCC to provide heterosubtypic influenza protection.

To understand how low-dose rapamycin specifically alters the expression of certain downstream mTOR effectors, we ran quantitative Polymerase Chain Reaction (qPCR) analyses on RNA isolated from PBS and rapamycin-treated B cells. Additionally, we ran western blots on PBS and rapamycin-treated B cells to investigate how low-dose rapamycin uses phosphoregulation to alter downstream mTORC1 protein expression. Based on our data, we found that rapamycin decreases expression of the *Slc2a1* gene, which encodes Glucose transporter 1 (GLUT1), and the phosphoprotein pS6, which helps encode B cell ribosome biogenesis. In addition, rapamycin may alter the expression of other genes, but to a lesser extent.

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A/HKx31	H3N2 influenza A virus
ACK	Ammonium-Chloride-Potassium Lysis Buffer
A.D.	Anno Domini
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
APC	Antigen-Presenting Cell
Bach2	BTB Domain and CNC Homolog 2 gene
B.C.	Before Christ
Bis-Tris	Bis-Tris Methane
BSA	Bovine Serum Albumin
BSL2	Biosafety Level 2
bZIP	basic Leucine Zipper Domain
Cx	Complement Protein x
CDC	Center for Disease Control
cDNA	complementary DNA
CD8	Cluster of Differentiation 8
c-Myc	Avian myelocytomatosis virus oncogene cellular homolog gene
CVF	Cobra Venom Factor
C57 BL/6J	C57 Black 6J mice
°C	degrees Celsius
DNA	Deoxyribonucleic Acid
ΔΔCT	Double Delta Threshold Cycle
EID_{50}	egg 50% infective dose

ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
Fc	Fragmented, crystallizable
FcR	Fragmented, crystallizable Receptor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC	Germinal Center
GLUT1	Glucose Transporter Protein 1
HxNx	Hemagglutinin x Neuraminidase x
HA	Hemagglutinin
Hif1α	Hypoxia-inducible factor 1 alpha subunit gene
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-4	Interleukin-4
Irf4	Interferon Regulatory Factor 4 gene
i.n.	Intranasally
i.p.	Intraperitoneally
LPS	Lipopolysaccharide
mAmp	milliAmp
mFcRIV	murine Fragmented, crystallizable Receptor IV
MHC	Major Histocompatibility Complex
mL	milliliter

mRNA	messenger Ribonucleic Acid
mTOR	mammalian Target of Rapamycin
mTORC1	mammalian Target of Rapamycin Complex 1
M2	Matrix-2 Proton Channel
NA	Neuraminidase
NFAT	Nuclear factor of activated T cells
NK	Natural Killer cell
nm	nanometer
NMS	Normal Mouse Serum
PBS	Phosphate-Buffered Saline
PDH	Pyruvate Dehydrogenase
Pdk1	Pyruvate Dehydrogenase Kinase 1 gene
PE	Purified anti-phycoerythrin antibody
PR8	A/Puerto Rico/8/34 virus
pS6	phospho S6 ribosomal protein
qPCR	quantitative Polymerase Chain Reaction
Rap/RAP	rapamycin
RIPA	Radioimmunoprecipitation Assay Buffer
RLU	Relative Light Units
RNA	Ribonucleic Acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RQ	Relative Quantification

Slc2a1	Solute carrier family 2 member 1
TBST	Tris Buffered Saline with Tween
µg	microgram
µL	microliter
V-DJ	Variable-Diversity Joining recombination
Vn1203	Vietnam 1203 virus
Xbp1	X-box Binding Protein
4E-BP1	4E-binding protein

LEGEND

*	Indicates significant differences in graphs

CHAPTER 1

INTRODUCTION

1.1: HISTORY OF INFLUENZA

The term "influenza" was born in 14th Century Italy as an explanation for the cause of disease, described by a variety of ailments whose appearances were believed to be dictated by the position, or influence, of the stars[1]. Though it is now accepted that the virus's history began much earlier and had little to do with the cosmos, unanimity regarding its exact origin remains elusive. One camp of medical historians believes that the first record of a true influenza epidemic resides in Book VI of Hippocrates' *On Epidemics* series[2]. Book VI describes a pestilence with symptoms commonly attributed to the influenza virus, such as fatigue, coughing, and fever, that swept the northern Greek port town of Perinthus in 412 B.C[3]. Proponents argue that these symptoms, and the correspondence of the outbreak with the winter solstice, provide strong evidence that Perinthus was plagued by influenza virus[2]. Others dispute that the book's marked inconsistencies with modern knowledge of influenza—namely, the presence of other symptoms not related to influenza, and the absence of pneumonia in relapse cases—cast sufficient doubt on the disease's identity[4]. Doubt also arises from the inherent potential for error from the secondhand authorship of the account. Unlike Books I, III, and V, which were written by the man himself, Books II, IV, VI, and VII were written by Hippocrates' son Thessalus using his father's notes as a guide[5]. Thus, others posit that the first likely influenza event came in the form of an epidemic that decimated French King

Charlemagne's army in 876 A.D. during his attempted conquest of Europe[6]. Still others only consider accounts written during or after the Middle Ages as reliable representations of true influenza events[6]. While many arguments exist, lack of hard data and sufficient corroboration have ensured that the search for influenza's origin remains speculative for the time being.

By the 16th Century, influenza had already earned a page in the history books with a series of devastating epidemics that altered the course of numerous wars and devastated entire countries at a time. However, the year 1510 marked the first recognized influenza pandemic[6,7]. Starting in Asia and quickly spreading to Africa and Europe, the pandemic demonstrated the true spreading potential of the influenza virus[7]. The next flu pandemic in 1557 further raised the stakes with significantly higher mortality rates than the 1510 pandemic[6]. The final pandemic of the 16th Century occurred during 1580, and spread over contiguous Eurasia, Africa, and possibly the Americas to kill 8,000 Roman citizens, entire Spanish towns, and countless others[8]. Almost as alarming as the death toll of the 1580 pandemic was the quickness with which it spread. Unlike the 1557 pandemic, whose death toll was spread out over the next few years, the 1580 pandemic killed all of its victims within the span of one year[6,9]. After a brief lull in the 17th Century, influenza pandemics would rise again and continue to sporadically arise, infect, and kill over the next two centuries. With the exception of a few outliers, the number of infected individuals remained within certain parameters, and the number of casualties rarely eclipsed a million. All this soon changed in the 20th Century with the arrival of an especially virulent strain that would mark the next turning point in the history of influenza.

Coming on the heels of the First World War, the infamous influenza pandemic of 1918 infected a third of the world's population and killed anywhere from 50-100 million people in what still stands as the deadliest single event in all of human history[6,10]. The colloquially-named Spanish Flu achieved this enormous level of destruction over the course of three successive infection waves that all occurred within a single year's time. The first and mildest of these waves was by most measures unremarkable; though its morbidity rates were somewhat high, its mortality rates fell well within the parameters of previous pandemics, giving no reason to fear any worse[10]. A few months later, the second wave of infection arrived without warning. Emerging first in southern England, the nightmarish outburst killed millions of victims and set new precedent for the lethal potential of an influenza pandemic[10-11]. Almost as astonishing as how many people it killed was whom it killed, and whom it didn't. Bucking the tradition of previous influenza pandemics that had primarily affected the oldest and youngest individuals, the second wave of the Spanish Flu plucked most of its victims from the least likely population: young adults between 20 and 40 years of age[9]. Although elderly and infant populations did experience relatively higher mortality rates than the general population during the second wave, young children and adults over 65 were surprisingly spared[12]. These trends gave rise to what became known as the W-shaped mortality curve, formed from mortality rate peaks in newborns, young adults, and the elderly[12]. The third and final wave of the 1918 pandemic was the only one to appear during influenza's usual winter season[10]. While milder than its bloodthirsty predecessors, the last chapter of the Spanish Flu still managed to cause thousands of deaths, both in previously hit countries

such as Spain, and in new countries such as Australia[13]. Fortunately, like its brethren before it, the last outburst subsided within a few months[10].

As the 1918 pandemic wound down to a close, the world was left ravaged and confused by the worst medical catastrophe it had ever known. Much remained unclear. Despite the recognition of influenza as the causative agent and extensive research efforts during the pandemic, scientists were still stumped as to what influenza actually was, and how it had caused so much destruction. The generally accepted theory at the time alleged that a bacterium known as *Bacillus influenzae*, which had previously been isolated from the nostrils of influenza victims, was the perpetrator behind the disease[14]. In spite of some conflicting evidence for this theory, the search for additional answers largely died down with the pandemic. Thus, another decade would pass before the true source was identified.

The first step to catching the deadliest serial killer of all time was taken unintentionally by Rockefeller Institute researcher Richard E. Shope in 1931. Shope and his mentor Paul Lewis were investigating a disease outbreak in Iowan pigs, which was dubbed "swine influenza" for its striking similarities to the human pestilence[15]. Expecting to find a bacterial cause of morbidity, the pair were surprised to discover that isolated bacterium alone did not induce disease[16]. Further experiments revealed that the real culprit behind initial infection was small enough to pass through a bacteria-resistant filter and caused mild infections on its own[17]. When combined with the isolated bacterium, however, the tiny perpetrator was found to induce the same serious condition initially seen in the affected pigs[17]. Therefore, Shope declared that severe swine influenza was the

result of a coinfection, a principle that would redefine the understanding of influenza pandemics.

Two years after Shope's invigorating discoveries, human influenza virus was isolated by Wilson Smith, Christopher Andrewes, and Patrick Laidlaw[18]. Using Shope's technique, the three scientists filtered throat samples from influenza patients of the 1933 epidemic through bacterially-impermeable material. The trio inoculated an initial cohort of ferrets with the filtrates after discovering their susceptibility to sickness through trial and error. After the first ferrets demonstrated obvious influenza symptoms with infection, their nostrils were scraped and emulsified for injection into other ferrets. In this way, 26 ferrets were infected with the same influenza virus, each from scrapings of the previous ferret. Even with potential dilution from multiple sample transfers, each ferret in the series still succumbed to the effects of the original filtrate. To prove the filtrate's infectious agent could not be a bacterium, the team stimulated the filtrates with a variety of bacterial cultures and growth factors. Not a single bacterium grew, and the scientists concluded, once and for all, that the source of influenza had to be a virus[18].

The published findings of Shope, Smith, Andrewes, and Laidlaw inspired many upcoming scientists to pursue careers in the exciting new fields of virology and influenza research[15]. The resultant boom in these fields yielded fifteen years of discovery that yielded a much enhanced characterization of the virus, and the development of its first vaccine[11]. Yet for all these advancements, man still lacks the proper tools to prevent and sequester influenza pandemics. The first evidence of this deficiency came in the form of the H2N2 influenza pandemic of 1957-58. Originating in China, the so-called Asian flu spread across the globe in a matter of 4 months[6]. Although vaccines were developed,

distribution delays and low efficacy prevented them from being a significant factors in the containment of H2N2[11,19]. Even with this setback, and two distinct waves of infectious attack, the mortality rate of the 1957 pandemic was comparatively mild to its 1918 predecessor. It is estimated that over the course of the entire pandemic, less than 2 million people were killed by the Asian strain[9,20]. The next exhibition of influenza's tenacity, the 1968 H3N2 pandemic, was similarly mild. Spread by air travel and Vietnam War veterans, the Hong Kong Flu infected people in two successive waves, killing about 2 million people[11,21]. The rapid spread and high mortality rates of the Asian and Hong Kong influenza strains demonstrated that, despite vaccine development, man was no closer to preventing influenza pandemics than he had been in 1918.

Named for its porcine genetic origin, the most recent and mildest influenza pandemic was the 2009 Swine Flu. In spite of shared protein subtypes with the Spanish Flu, and a similar disproportionate attack on those under 65 years of age, the 2009 H1N1 pandemic only caused an estimated 575,400 deaths globally[22]. It is not known whether the reduced death toll of this pandemic was due to human ingenuity, or viral characteristics. Some facts are evident, however. Even though the 2009 pandemic had a reduced death toll, humanity once again failed to prevent another pandemic with modern viral combative tools. Thus, if another pandemic were to arrive tomorrow, man would still be unable to prevent it.

1.2 CHARACTERIZATION OF THE INFLUENZA VIRUS

All influenza viruses belong to the Orthomyxoviridae family of antisense ssRNA viruses[23]. From the shared Orthomyxoviridae family, influenza viruses diverge into

influenza types A, B, C, and D based on their host range and overall severity. Although types A, B, and C infect humans, only types A and B are capable of causing severe disease and epidemics, and only type A is capable of causing pandemics[24]. Thus, only influenza types A and B are of clinical significance, and have vaccines developed against them. Each type acquires a protective lipid envelope during intracellular assembly that is derived from the lipid bilayer of the host cell[23,25]. This borrowed bilayer houses three surface proteins of significance: the glycoproteins hemagglutinin (HA) and neuraminidase (NA), and the Matrix-2 (M2) proton channel[26]. Of these three proteins, HA and NA are the most clinically significant due to their key pathogenic functions and overall prevalence on the viral surface. HA constitutes 80% of the viral surface proteins, and promotes cellular infection by coordinating the attachment to and penetrance of a host cell[27]. NA makes up 17% of viral surface proteins, and promotes infection spread by coordinating cellular exit[27]. Thus, HA and NA subtypes define an influenza strain, and are the primary antigenic targets of the immune system's antibody response.

Due to pressure from the immune response, recurrence of influenza requires mutations in HA and NA sufficient to evade immunological memory. Most of these mutations arise from errors during viral RNA replication. Unlike DNA, RNA cannot repair its own mutational errors, so these changes accumulate over time[28]. Those strains that accumulate enough mutations to evade the immune response are able to spread efficiently, which is known as antigenic drift.

Although mutated viruses gain new antigenic glycoprotein subtypes, some segments of the original glycoprotein structure are often conserved. When exposed to a virus with a certain set of conserved glycoprotein segments, the immune system will

develop a degree of cross-protection against all viruses with those segments. Since antibodies are generated against these structures, antibodies previously generated against a certain glycoprotein structure may exhibit some affinity for glycoproteins on other influenza viruses with similar structures. Thus, conserved glycoprotein segments can mediate the induction of a limited secondary immune response, which leads to cross-protection against similar mutated influenza viruses. While this cross-protection alone is not enough to prevent an influenza infection, it can reduce its overall severity. The conferrable pathogenicity of glycoprotein mutation is thusly limited. With this mechanism as its sole method of adaptation, influenza B viruses are incapable of becoming highly pathogenic pandemic viruses. For this reason, and the greater prevalence of human influenza A viruses, the majority of vaccines are designed to protect against influenza A strains.

The spread of highly pathogenic influenza A strains, such as the H5N1 and H7N9, in birds, poses a new threat for a pandemic. Most viruses from this avian reservoir cannot efficiently infect humans due to differences in host cell binding sites: the HA of avian viruses binds sialic acids with α (2, 3) galactose linkages, while the HA of human viruses binds sialic acids with α (2, 6) galactose linkages[27]. However, mutations in the avian viruses can enable them to bind to α (2, 6) galactose linkages. In addition, multiple viruses can simultaneously infect an animal and mix their genes to make completely novel viruses[28]. These rare events, known as an antigenic shifts, result in the creation of new, highly pathogenic viruses that can cause global infections due to lack of previous immunity in the human population[29]. Thus, it is access to the avian reservoir that confers influenza A viruses with pandemic potential. Although the H5N1 and H7N9 avian strains

do not transmit efficiently between humans, several human infections with these viruses have been confirmed. Of these confirmed infections, 40% of those infected with H7N9 and 60% with H5N1 have died[30-31]. Thus, if these viruses mutated so that they could transmit efficiently between humans and maintained their high mortality rate, millions of people would die.

1.3: MODALITY AND LIMITATION OF THE SEASONAL INFLUENZA VACCINE

The most effective defense against influenza infections is vaccination[32]. Current vaccines prevent infection by stimulating the production of specific, neutralizing antibodies against the HA and NA glycoproteins of preselected viral strains. These antibodies bind to the HA and prevent the virus from binding receptors on the host epithelial cells, or bind the NA and prevent the spread of the virus from one cell to another. Neutralizing antibodies are produced following vaccination after viral antigens are processed and transported to the lymph nodes by antigen-presenting cells (APCs), namely dendritic cells and macrophages[33,34]. In the lymph nodes, APCs activate B cells and T cells by displaying these antigens to specific receptors on each cell. Activated B cells internalize, modify, and display the antigen via MHC II (Major Histocompatibility Complex) receptors, which in turn activate T cells[34]. Upon meeting and binding, activated B and T cells form structures in the lymph nodes known as germinal centers (GCs). In the GC, antibodies that bind to the antigen with the highest affinity are selected to develop through a process known as somatic hypermutation. In addition, in the GC, antibodies undergo class switch recombination and switch from the IgM to the IgG

isotype, which allows the antibodies to acquire specific functions[34]. These processes heighten the specificity of B cell antibodies against vaccine glycoprotein antigens through modification of variable and constant antibody regions[33,35]. Following the GC reaction, the B cells proliferate extensively. Although most activated B cells become antibody-secreting plasma cells, some B cells differentiate into memory B cells to initiate a quick immune response should the virus be encountered again. It is the production of these memory cells with specific, neutralizing antibodies that confers protection against infection with the influenza strains represented by the vaccine.

Unfortunately, the seasonal influenza vaccine is not without its weaknesses. Perhaps the most glaring of its insufficiencies is its inevitable obsolescence. Due to the constant antigenic drift of the virus, the vaccine of the previous influenza season will become outdated by the next one, if not long before. Thus, the vaccine must be updated each season to reflect whichever strains researchers predict to be most prevalent at the start of production[36]. This flaw in itself would not be so egregious if it were not for the length of time required to produce the vaccine. As it stands, the most common method of mass production involves growing the virus in fertilized hen's eggs[37]. While the viral egg growth only takes three weeks, the necessary optimization, purification, regulation, and testing extends the total production time to about five or six months at best[38]. The large gap between identification of strains and distribution of vaccines gives the virus more time to mutate and potentially outpace the vaccine. In this way, the current method of production diminishes both specific influenza vaccine efficacy and general influenza vaccine faith.

A host of other factors further reduce the vaccine's protective ability by limiting its general efficacy. Vaccine effectiveness varies with both demographic and viral characteristics. In human populations, vaccine efficacy is affected by age and health. Young, healthy people generally have stronger immune systems and typically respond better to vaccines than the elderly or individuals with chronic disease[36]. The viral subtype also impacts vaccine effectiveness. A meta-analysis conducted from 2004-2015 found the effectiveness for each vaccine subtype to be as follows: 33% for H3N2 viruses; 54% for Influenza B viruses; and 61% for H1N1 viruses[39]. With data from independent studies such as this one and its own internal research, the CDC estimates that, at best, the vaccine can only reduce the risk of contracting influenza infection by 50-60% on average[36].

More disconcerting than its shortcomings in the fight against seasonal influenza infections is this the current vaccine's inherent inability to provide proper protection in the event of a pandemic. Most influenza pandemics have run to completion within the span of a single year[6]. Many of these, including the especially lethal pandemic of 1918, have incurred the majority of their morbidity and mortality in merely two to three months over a single wave of infection[10]. At the current pace of mass production, a viable vaccine would not be ready in time to prevent or treat such a strain, leaving the global population essentially defenseless against it. Assuming the vaccine was produced in time to confer some benefit, it would likely only come to the developed world, while limits in distribution would likely leave the developing world high and dry until the pandemic had already passed[40]. The best case scenario in such an event would be a continuation of the trend established in the last three pandemics: widespread morbidity, relatively low mortality, and inevitable economic disruption from mass sickness. Yet there is no

guarantee that this pattern will persist. Should a novel viral strain arise from the avian reservoir, humanity must be ready, or suffer incalculable consequences.

It should be noted that while the influenza vaccine is the most effective treatment option against influenza infection, it is not the only one available. Active treatments also exist in the form of antiviral medications. Although these drugs can reduce the duration of an influenza infection by one to two days, which can make a large difference in patients with certain disorders, they are not sufficient to cure or prevent infection[41]. Furthermore, influenza antivirals do not work against all strains; since their mechanism of action is based on NA inhibition, mutations or recombinations in NA can inhibit antiviral therapy[42]. Thus, influenza antivirals are not a viable solution in the event of a pandemic virus, and are only mildly effective against seasonal epidemic viruses.

1.4: RAPAMYCIN: A POTENTIAL MODEL FOR A UNIVERSAL INFLUENZA VACCINE

Despite available therapies, influenza causes 250,000-500,000 deaths per year[43]. Given this, and our practical defenselessness against highly pathogenic and potentially lethal pandemic influenza viruses with current treatments, it is evident that a new remedy will be necessary to conquer influenza. The most pursued path to influenza eradication in recent years has been the search for a universal influenza vaccine. Theoretically, a universal vaccine would provide immunity against all subtypes of influenza—a concept known as heterosubtypic immunity—by producing neutralizing antibodies against epitopes conserved in all subtypes of influenza, including highly pathogenic pandemic strains. Although antibodies cross-reactive against all HA subtypes have been discovered

in humans, these antibodies are extremely rare and insufficient production of corresponding memory cells prevents their establishment of heterosubtypic immunity[44–46]. Thus, an effective universal influenza vaccine will require strong stimulation of both conserved cross-reactive antibodies and sufficient corresponding memory cells.

In 2013, our lab discovered that mice treated with a low dose of the immunosuppressant, rapamycin, during influenza vaccination, had increased levels of cross-reactive influenza antibodies[47]. Consequently, rapamycin-treated mice were protected against subsequent infections with multiple subtypes of influenza. Traditionally used at higher doses as a prophylactic to prevent renal transplant rejection, rapamycin suppresses the immune system by inhibiting a regulatory serine/threonine kinase known as mTOR[48,49]. mTOR mediates cell survival, metabolism, and proliferation in response to extracellular stimuli such as nutrients, growth factors, and antigen[50,51]. Surprisingly, Araki et al. found that treatment of mice with a low dose of rapamycin (75 μg/kg) actually boosted production of memory $CD8^+$ cytotoxic T cells[52]. Since $CD8^+$ T cells dominate the immune response to the internal, conserved influenza proteins, we tested whether rapamycin could enhance heterosubtypic immunity in a mouse model[53,54]. Mice were infected intraperitoneally with an H3N2 influenza virus (HKx31). Because influenza does not replicate outside of the respiratory tract, infection via this route allows the virus to produce the full spectrum of proteins without an infection, and therefore serves as a model of vaccination. After 28 days, mice were challenged by an intranasal infection with a H5N1 strain (Vn1203). In addition, one group of mice was treated daily with 75 μg/kg of rapamycin beginning one day prior to vaccination and ending the day before the challenge. In mice treated with a Phosphate-Buffered Saline (PBS) control,

vaccination with H3N2 did provide some protection, however 70% of the mice died following the H5N1 infection. In contrast, less than 10% of the mice treated daily with rapamycin died following the H5N1 infection, indicating that rapamycin significantly increases heterosubtypic immunity to influenza virus[47]. Through further investigation, it was discovered that rapamycin enhanced survival by altering the antibody response during the vaccination. Rapamycin diminished germinal center formation to effectively reduce antibody class switching. As a result, rapamycin-treated mice had higher levels of influenza-specific IgM antibodies, as well as antibodies with distinct specificities than control-treated mice. We use rapamycin treatment as a model to understand what parameters of the immune response confer optimal heterosubtypic immunity.

1.5: AIMS AND OBJECTIVES

Although it is known that rapamycin inhibits mTOR, it is not understood how a partial block in this pathway provides optimal heterosubtypic immunity. Furthermore, it is not clear which pathways downstream of mTOR are blocked with the low dose of rapamycin used in our model. Additionally, we do not understand how the antibodies generated in the presence of rapamycin enhance protection against distinct subtypes.

The first aim of my project was to investigate how antibodies generated in the presence of rapamycin provide heterosubtypic immunity. We specifically investigated whether these antibodies required the use of the complement system or antibody-dependent cell-mediated cytotoxicity (ADCC) to provide such protection. To test the role of complement, we inhibited the complement system *in vivo* using Cobra Venom Factor (CVF), and compared the efficacy of rapamycin treatment between control and CVF-

treated mice following H5N1 infection. To examine ADCC, we depleted natural killer (NK) cells, which mediate ADCC, and tested whether rapamycin treatment enhances heterosubtypic immunity in their absence. We also performed an *in vitro* ADCC assay with serum from PBS or rapamycin-treated mice. Based on our data, we concluded that rapamycin-mediated antibodies do not use the complement system or ADCC to provide heterosubtypic influenza protection.

The second aim of my project was to understand how low-dose rapamycin specifically alters the expression of certain downstream mTOR effectors. To this end, we ran quantitative Polymerase Chain Reaction (qPCR) analyses on RNA isolated from PBS and rapamycin-treated B cells. Based on our data, we found that rapamycin decreases expression of the *Slc2a1* gene, which encodes Glucose transporter 1 (GLUT1). In addition, rapamycin may alter the expression of other genes, but to a lesser extent.

CHAPTER 2

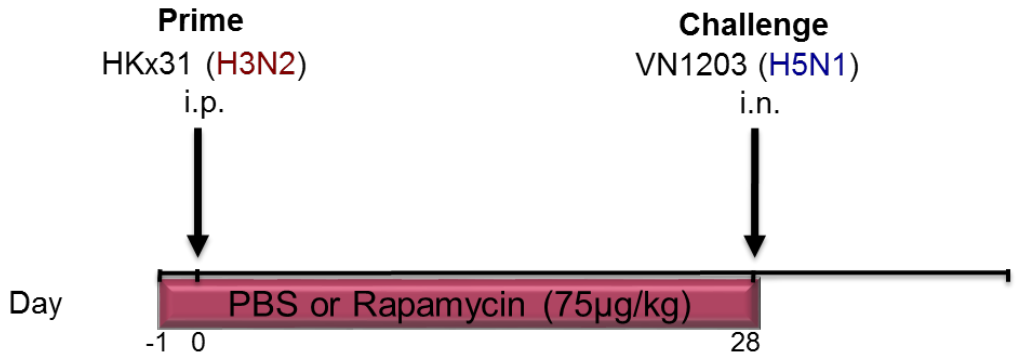
MATERIALS AND METHODS

2.0: GENERAL *in vivo* TREATMENT MODEL

Mice

Female 8-10 week old C57 BL/6J mice were purchased from the Jackson Laboratory and held under specific pathogen-free conditions at St. Jude Children's Research Hospital. All *in vivo* experiments used this breed of mice unless otherwise noted.

Low-Dose Rapamycin Treatment Protocol

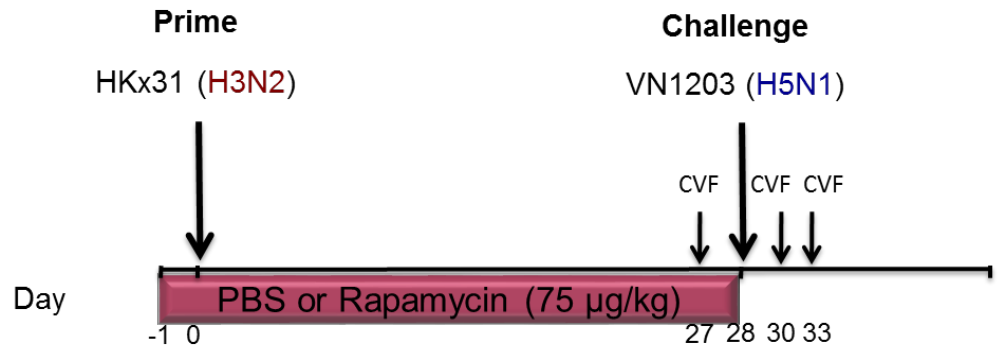


Supplemental Figure 2-1: General Rapamycin Treatment Model

All *in vivo* experiments were planned using the following treatment template: On Day -1, mice receive their first assigned treatment of either 0.1 mL Phosphate-Buffered Saline (PBS), or low-dose rapamycin (Rap)—defined here as a concentration of 75 µg/kg of body weight diluted in PBS—via intraperitoneal injection (i.p.). On Day 0, mice are infected i.p. with of 1 x 10^8 egg 50% infective dose (EID_{50}) of HKx31 virus, which

contains 6 internal genes of A/Puerto Rico/8/34 (PR8) and H3 and N2 glycoproteins. This virus undergoes a defective growth cycle in which all viral proteins are produced without infectious virus to mimic a vaccine-like effect. Following 28 more days of assigned treatment, mice are challenged with an intranasal (i.n.) infection of 4.5 x 10^5 EID_{50} of A/Vietnam/1203/04 virus, a lethal dose. This H5N1 virus contains the internal PR8 genes, and was modified for use in a BSL2 facility. Survival and weight loss data are then recorded over the next 10-12 days.

2.1: CVF EXPERIMENT



Supplemental Figure 2-2: CVF Treatment Model

Infection and Observation

A cohort of 40 mice was divided equally into four treatment groups: PBS (Phosphate-Buffered Saline), PBS + CVF, Rap, and Rap + CVF. PBS and Rap treatments followed the general treatment model through Day 28 with some modifications. On Day 27, a portion of the mice were given 30 µg CVF (Millipore Product #233552) to initiate complement depletion. These mice received the same dose of CVF every 3 days after the initial dose to maintain steady depletion throughout the post-infection challenge data collection period, according to the protocol established by Benhnia et. al (**Supplemental**

Figure 2-2)[55]. Following the ΔVn1203 (H5N1) infection challenge, percent survival and percent weight loss were recorded. Data graphing and statistical analysis were completed using GraphPad Prism version 5.0. Survival experiments were analyzed using the Kaplan-Meier survival probability estimates. Weight loss comparisons were made using unpaired *t* tests. Quantitative differences between two samples were compared with the Mann-Whitney *U* test. *P*-value < 0.05 was considered significant.

ELISA

An ELISA performed to assess the depletion of complement by CVF was performed using the Novateinbio C3a ELISA Kit (#NB-E20368). The ELISA was performed following the kit's protocol.

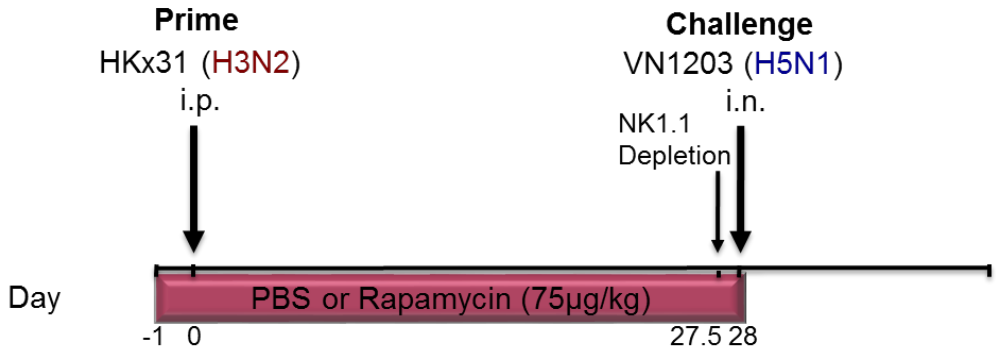
2.2: ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY EXPERIMENT

2.2a: RELATIVE QUANTIFICATION OF Fc RECEPTOR BINDING

Fc receptor binding *in vitro* was relatively quantified in infected target cells using the Promega mFcγRIV (murine Fragmented, crystallized gamma receptor 4) Reporter Bioassay Kit (Promega M1201). The assay was performed according to the manufacturer's protocol. Target cells were infected with A/HK/x31 or A/Vietnam/1203/4, and incubated with serum from a previous low-dose Rap treatment model, described above. Serum was heat treated to remove the impact of complement.

2.2b: ADCC SURVIVAL AND WEIGHT RATES

Mice followed the general treatment model for PBS and Rap treatments (**Supplemental Figure 2-1**). Twelve hours prior to the challenge infection, a portion of the mice were given anti-NK depleting antibody (PK136) (**Supplemental Figure 2-3**).



Supplemental Figure 2-3: Treatment Model for NK1.1 Depletion

Weight and survival data was monitored daily. Data graphing and statistical analysis was done using GraphPad Prism version 5.0. Survival experiments were analyzed using the Kaplan-Meier survival probability estimates. Weight loss comparisons were made using unpaired *t* tests. Quantitative differences between two samples were compared with the Mann-Whitney *U* test. *P*-value < 0.05 was considered significant.

2.3: QUANTITATIVE POLYMERASE CHAIN REACTIONS (qPCRS)

General B cell Purification

Spleens were collected, mashed with screens, and spun down at 600 xg for 5 minutes. After their red blood cells were lysed with 5 mL of 1X ACK Lysis Buffer for 2 minutes, the leftover cells were topped with PBS + 2% serum and spun again. The cells were then resuspended in 5 mL PBS + 2% serum and counted in 0.04% Trypan Blue in PBS with a hemocytometer. After saving $1x10^6$ cells for a purity check, a single-cell-

suspension of lymphocytes at a concentration of $1x10^8$ cells/mL was made in separation buffer. The cells were then transferred into 12x75 mm, 5 mL tubes at $2x10^8$ cells/tube. B cells were negatively selected using the MagniSort Mouse B cell Enrichment Kit (eBioscience, 8804-6827).

Setup

After purification, the B cells were resuspended in RPMI at $4x10^6$ cells/mL and plated in a 24 well plate at $2x10^6$ cells/well. B cells were given 500 µL of just media, media with LPS (5 µg/mL) and IL-4 (10 ng/mL), or media with LPS (5 µg/mL), IL-4 (10 ng/mL), and Rap (0.5 ng/mL) for 0, 24, or 48 hours of stimulation. At each time point, B cells were washed, centrifuged, and lysed in Buffer RLT with 2-Mercaptoethanol (1mL:10µL). To ensure complete mixing and lysis, the lysed B cells were vortexed and mixed thoroughly with a blunt needle.

RNA Isolation

RNA isolation was performed using a Qiagen RNeasy minikit according to the manufacturer's protocol (#74104).

RNA Quantification

Eluted RNA was quantified using a Nanodrop 8000 system. RNA quantities, 260 nm/280 nm absorbance ratios, and 260 nm/230 nm absorbance ratios were recorded. Sample quality was determined by comparing recorded 260/280 values to the generally accepted RNA 260/280 ratio of 2. The unstimulated sample with the 260/280 value

furthest from this ratio was excluded from the study. Stimulated samples with 260/280 ratios greater than 2.20 were also excluded.

cDNA Synthesis

For each viable sample, volumes containing 750 ng of RNA were calculated and plated into a 96 well plate. Each sample well received 4 µL of 5X VILO Reaction Mix and 2 µL of 10X Superscript Enzyme Mix, each from a ThermoFisher Superscript VILO cDNA synthesis kit. Wells were then brought up to 20 µL with DEPC treated water. cDNA was synthesized in a Veriti 96 Well Fast Thermocycler in three stages: 10 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 85°C.

qPCR Plate Preparation

Newly synthesized cDNA samples were transferred onto a 96 well plate and diluted with enough RNase-free water to bring the total volume of each well to 14 µL. The diluted cDNA samples were then aliquoted onto a new 96-well plate in 2.5 or 3 µL duplicates. Each sample and duplicate plate received 10 µL of qPCR master mix, 1 µL of primer for the gene of the transcription factor being tested, and enough RNase-free water to bring the total volume up to 20 µL. This process, duplicates included, was repeated on the bottom of the plate with a primer for GADPH as an endogenous control for the qPCR. Plates were covered with adhesive plastic and either run immediately after or stored covered on ice until they could be run.

qPCR and Statistical Analysis

All qPCR's were run on a standard protocol on a 7900HT qPCR instrument (Applied Biosystems). The data were analyzed using the RQ manager v1.2 software (Applied Biosystems) applying the ΔΔ CT method. The relative gene expression values obtained were plotted and statistical analysis carried out using GraphPad Prism. These differences were considered significant at the p=0.05 level.

2.4: WESTERN BLOTS

B cell Isolation and Purification

B cells were isolated as above.

Cell Stimulation and Plating

The B cells were resuspended into 3 mL of RPMI media, and plated into 6 well plates at 50 x 10^6 cells/well. Cells were given 0.5ng/mL Rap, or media alone, for 2 hours followed by stimulation with LPS (5 μg/mL) and IL-4 (10 ng/mL) for 0, 1, 6, 24, and 48 hours. At each time point, cells were pelleted and frozen for future analysis. The 48 hour Rap-treated B cells received an additional Rap spike of 1 ng/well after 24 hours to account for the half-life of rapamycin.

BCA Protein Assay and Protein Quantification

The stored B cells were put on ice and lysed with 45 μL ice cold RIPA buffer containing phosphatase and protease inhibitors for 30 minutes. The lysate was centrifuged at 20,000+ rpm for 5 minutes and the supernatant collected, and put on ice.

Protein was quantified via BCA Protein Assay. BCA Protein Assay was performed according to the manufacturers protocol (Pierce 23227).

Membrane Preparation and Imaging

Loading dye, sample buffer, and 30µg of lysate were combined and boiled prior to loading on the gel. The samples were then run on a Bis-Tris gel for 90 minutes at 150 volts, and transferred to a nitrocellulose membrane at 250 milliamps (mAmps) for 1 hour. Membranes were blocked in 5% w/v BSA for 1 hour prior to overnight incubation with primary antibody (1:1000) at 4 °C. The membrane was washed and then incubated with Anti-rabbit IgG, HRP-linked secondary antibody (1:5000) in 5% w/v BSA in 1X TBST 1 hour at room temperature. After incubation, the membrane was washed.

Equal parts of Detection Reagent 1 and Detection Reagent 2 were mixed in a tube and vortexed to make developer. Developer was aliquoted onto a sheet protector, and a blot was placed down into it. Developer was aliquoted on top of the blot, the sheet protector was closed, and the blot was allowed to incubate for 4 minutes. The blots were placed in a new sheet protector with care taken to smooth out any bubbles before imaging in an Amersham Imager 600. After completing this process for phospho-antibody imaging, these blots were stripped , blocked, and probed for total protein following the above procedure.

2.5: LPS vs. αIgM STIMULATION FLOW CYTOMETRY

B cell Isolation and Purification

B cells were isolated as above.

Cell Plating

Once the total number of B cells was counted, they were resuspended in RPMI media at $1x10^6$ cells/mL. The cells were plated in a 96 plate well at $1x10^5$ cells (100 µL)/well in triplicates for each time point. The cells were pretreated as above. After incubating at 37°C for 2 hours, the plate was spun at 600xg for 5 minutes, and the supernatant removed. The 0 hour time point wells received 200µL of media alone and were subsequently transferred to pre-labeled Eppendorf tubes. Appropriate stimulation was then added to each remaining plate. Stimulation wells were plated in triplicates as follows:

1. 200 µL of media with LPS (5ug/mL) + IL-4 (10ng/mL) at time points 1, 6, 24, and 48 hours
2. 200 µL of media with LPS (5ug/mL) + IL-4 (10ng/mL) + Rap (0.5ng/mL) at time points 1, 6, 24, and 48 hours
3. 200 µL of media with anti-IgM (10ug/mL) + IL-4 (10ng/mL) at time points 1, 6, 24, and 48 hours
4. 200 µL of media with anti-IgM (10ug/mL) + IL-4 (10ng/mL) + Rap (0.5ng/mL) at time points 1, 6, 24, and 48 hours

Cells were incubated at 37° C for indicated time points.

Cell Collection

At their collection times, the cells and their media were transferred to a pre-labeled Eppendorf tube, and 200 µL of pre-heated fix buffer was added. Tubes were incubated in a water bath at 37°C for 10 minutes. After fixing, the tubes were centrifuged at 600xg for 5 minutes at 4° C. Supernatant was aspirated and replaced with 300 µL cold FACS Buffer. The 0 and 1 hour time point samples were left on ice until the 6 hour time point was harvested and fixed.

Once all time points were fixed, samples were washed, and their supernatant aspirated. Perm buffer was added to each sample and transferred to a new 96 well plate that incubated on ice in the dark for 30 minutes. The plate was then spun down at 600xg for 5 minutes at 4°C. After all supernatant was removed, the plate was washed. This process was then repeated again with 100 µL of stain mix consisting of B220 to identify B cells, and either pS6 (Phospho-S6 Ribosomal Protein (Ser235/236) Antibody #2211) or p4E-BP1 (Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855) as applicable. Single color controls were also stained in this way at this time. Fluorescently-tagged antibodies to 650, PE, FITC, all 3, and a blank sample were added and incubated on ice for 25 minutes. After incubation, the samples were washed and resuspended in 100uL of FACS buffer for analysis. After all samples had been collected, they were analyzed on a flow cytometer.

CHAPTER 3

RESULTS

3.1: THE ROLE OF COMPLEMENT IN HETEROSUBTYPIC IMMUNITY

The complement system refers to a collection of serum proteins that work together to target and destroy pathogens in the blood. Through a cascade of stepwise cleavages, these proteins are converted to their active forms that induce pathogen death using a variety of pathways. Using the classical pathway of activation, the complement system can play a role in the elimination of virally-infected cells through cooperation with the adaptive immune system. As antibodies bind to viral epitopes, the formation of an antibody-antigen complex stimulates the exposure of a binding site for the first protein of the complement system, known as C1, on the antibody[56]. When C1 binds this antibody, the complement cascade begins. A few cleavages later, a cleaved C3 protein known as C3b can bind the Fc portions of the antibodies in the antigen-antibody complexes[56]. Phagocytes with C3b receptors can then engulf the entire infected cell and destroy it.

We found that rapamycin enhanced heterosubtypic immunity by altering the antibodies generated during vaccination, such that these antibodies were more effective against influenza strains of different subtypes compared to antibodies generated in PBS control-treated mice. However, the antibodies generated in the rapamycin-treated mice were not neutralizing. In other words, these antibodies did not function by preventing the challenge virus from binding to host epithelial cells. Therefore, we investigated other

mechanisms by which these antibodies could enhance protection against multiple influenza subtypes. To determine whether the antibodies generated in the rapamycin-treated mice utilized the complement system to provide heterosubtypic influenza protection, we depleted the complement system *in vivo* using cobra venom factor (CVF). CVF inhibits the complement system by forming an immune complex that functions as a C3/C5 convertase and resists normal methods of degradation[57]. This convertase continually cleaves 100% of C3 and C5 proteins to eventually deplete complement activity[58].

To examine the role of complement *in vivo*, we vaccinated the mice with HKx31 (H3N2) virus as described previously, and waited 28 days to challenge with an H5N1 virus. Half of the mice were treated with rapamycin and half with PBS. In addition, half of each PBS and Rap group was treated with CVF one day prior to infection, and every third day after that. To verify that complement was depleted, we performed an enzyme-linked immunosorbent assay (ELISA) testing the serum levels of C3 in each group of mice. The lack of C3 in serum from CVF-treated mice confirmed depletion (**Figure 3-1**).

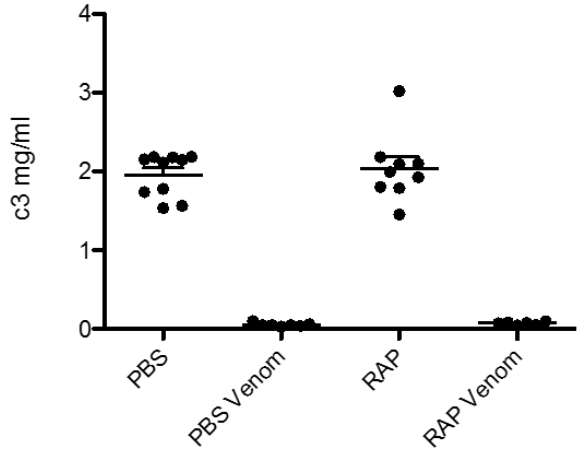


Figure 3-1: C3 ELISA Concentrations

Interestingly, the depletion of complement increased mortality in both the PBS and the rapamycin-treated mice (**Figure 3-2**). However, the rapamycin-treated group still showed enhanced protection compared to the PBS control group, suggesting that rapamycin is enhancing survival through a mechanism independent of complement. Had rapamycin used the complement system to enhance protection, survival rates in complement-depleted PBS and RAP mice would have been similar. The 57% difference in survival between the two suggests that this was not the case (**Figure 3-2**).

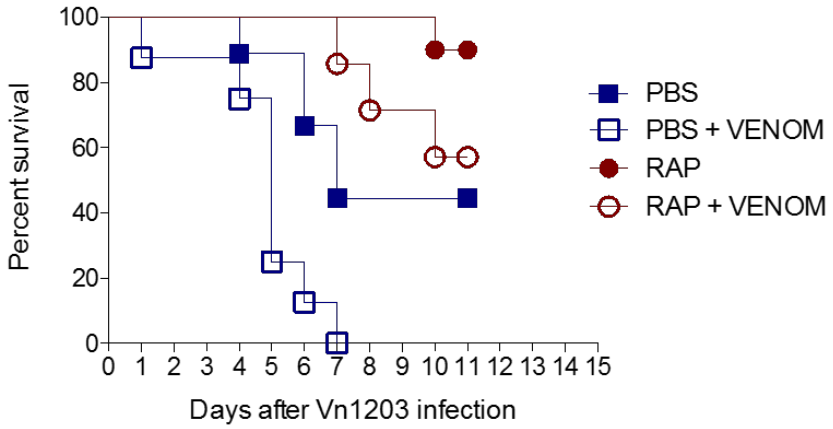


Figure 3-2: Percent Survival after Vn1203 Infection Challenge

3.2: THE ROLE OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC) IN HETEROSUBTYPIC IMMUNITY

Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) utilizes both the innate and adaptive immune systems to directly kill infected cells[33]. When a cell becomes infected with influenza, antigens of the specific influenza strain are presented on the surface of the infected cell, where they can be bound by an antibody whose variable region matches the antigen's structure. Once the variable region is bound to its antigen, a

portion of the antibody's constant region known as the Fc (Fragment, crystallizable) region can be bound by an Fc receptor (FcR) on natural killer (NK) cells. Upon binding, NK cells release cytotoxic molecules such as free radicals, perforins, and granzymes to kill the influenza-infected cell[33].

To determine whether antibodies generated in the rapamycin-treated mice utilize ADCC to provide heterosubtypic influenza protection, we used an *in vitro* ADCC assay that measures the ability of antibodies to bind viral antigens and activate NK cells. Mice were vaccinated with the H3N2 strain and given either rapamycin or PBS. Serum was collected on day 28 following vaccination to measure the optimal antibody response. FcR binding specific for ADCC was measured *in vitro* using a specially-designed line of effector cells that express the primary murine ADCC receptor, mFcγRIV, on their surfaces, and a downstream luciferase gene. Binding to mFcγRIV by viral antibodies activates a signal transduction pathway that induces downstream activation of the luciferase gene via the calcium-mediated activation of the nuclear factor of activated T cells (NFAT) transcription factor. Luciferase activation produces a bioluminescence effect that can be relatively measured and quantified in relative light units (RLU). We measured the ability of the antibodies to mediate ADCC against the H3N2 strain they were vaccinated with, and the H5N1 strain they were challenged with. The antibodies that bind the H5N1 infected cells are cross-reactive antibodies. As a negative control, we measured binding of the antibodies to uninfected target cells. Additionally, we included serum from a naïve mouse (NMS) that has never been infected with influenza as another negative control to confirm that the activity that we observed was due to influenza-specific antibodies.

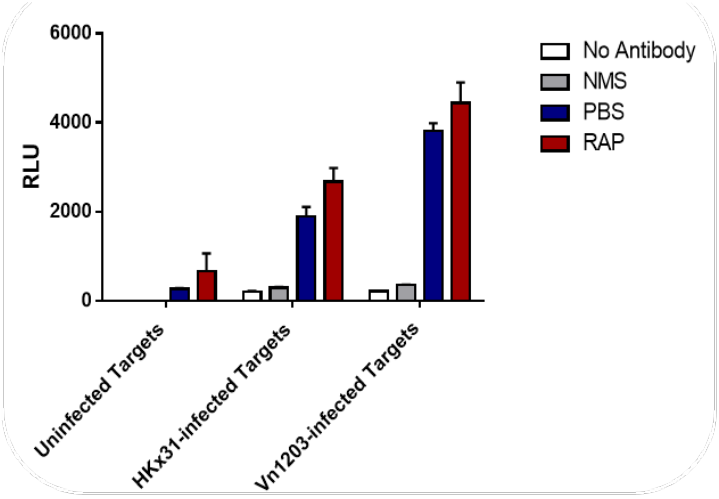


Figure 3-3: mFcRIV Binding in PBS vs rapamycin-treated cells

As Figure 3-3 illustrates, serum from PBS and rapamycin-treated mice vaccinated with H3N2 contained more antibodies that mediated ADCC compared to naïve mice, demonstrating that our system was working. In addition, there was little antibody binding to uninfected targets. Interestingly, we detected no significant difference in FcR binding were detected between the serum of PBS and rapamycin-treated mice for either virus tested, although both groups showed elevated binding over normal mouse serum (NMS). While the antibodies of rapamycin-treated mice appear to bind mFcγRIV slightly more than the antibodies of PBS-treated mice, the difference is not significant, suggesting that rapamycin does not enhance protection by providing enhanced ADCC activity. However, these data do not indicate whether ADCC is required for the rapamycin-mediated protection or whether the antibodies in the rapamycin-treated mice are binding different viral epitopes compared to control-treated mice.

To test whether rapamycin-enhanced protection requires ADCC *in vivo*, we depleted NK cells in PBS and rapamycin-treated mice and compared their survival and weight loss rates to isotype-treated mice.

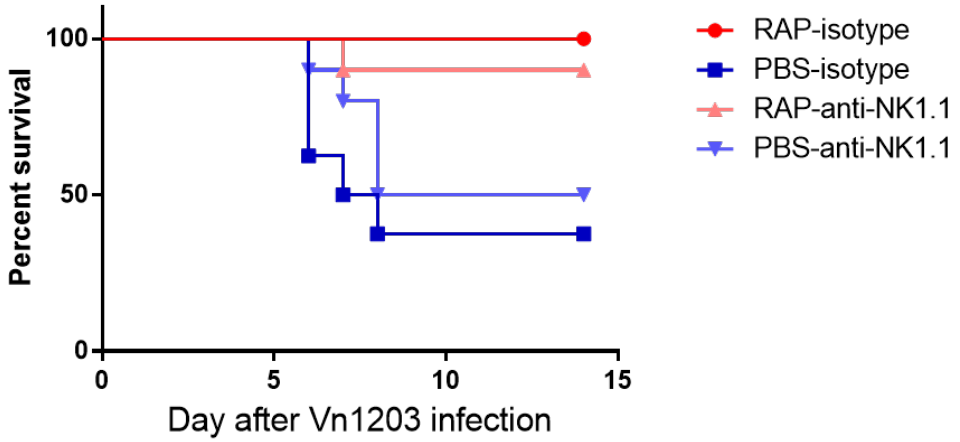


Figure 3-4: Survival Rates of NK1.1 Depleted Mice

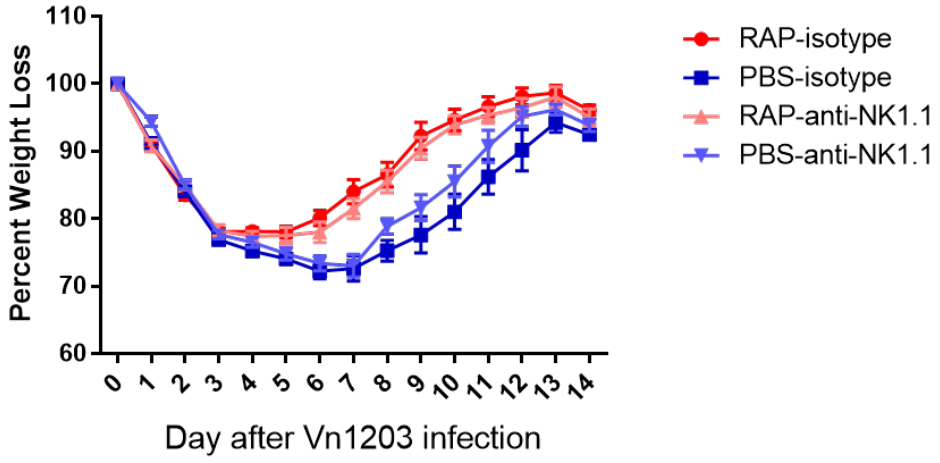


Figure 3-5: Percent Weight Loss of NK1.1 Depleted Mice

The near indistinguishability of the survival and weight-loss curves for isotype controls and NK-depleted mice indicates that ADCC activity is not required for rapamycin-mediated heterosubtypic protection (**Figures 3-4, 3-5**). Together, these results

with those of the FcR binding experiment suggest that rapamycin does not use ADCC as a means to enhance heterosubtypic immunity.

3.3: THE ROLE OF LOW-DOSE RAPAMYCIN IN B CELL GENE EXPRESSION (qPCRs)

To investigate how low-dose rapamycin specifically alters the expression of certain downstream mTOR effectors in B cells, we analyzed the expression of genes known to be downstream of mTOR. qPCR quantifies gene expression by probing isolated cellular mRNA for specific gene sequences, and amplifying these sequences against a control gene to relatively quantify expression across different treatment conditions. The genes we tested encode transcription factors that regulate cellular energy production and antibody specificity. While it is known that mTOR is required for the expression of many genes, we did not know to what extent rapamycin was blocking these pathways given the low dose of rapamycin that we used in our experiments. These data will inform us which pathways may be important for generating optimal cross-reactive antibodies.

3.3a: *Pdk1*

The Pyruvate Dehydrogenase Kinase 1 (*Pdk1*) gene codes for the production of pyruvate dehydrogenase kinases, which serve as regulators of aerobic respiration[59]. A crucial step of aerobic respiration is the conversion of pyruvate to acetyl-CoA by a multienzyme complex known as pyruvate dehydrogenase (PDH). By phosphorylating PDH, pyruvate dehydrogenase kinases can inhibit its activity, which ultimately results in the reduction of cellular energy production. In addition, previous studies indicated that

PDK1 regulates variable-diversity joining (V-DJ) recombination of both the heavy and light antibody chains in B cells[60-61]. V-DJ recombination promotes antibody specificity to combat the wide variety of antigens the immune system may encounter, and is necessary for B cells to generate antibodies specific for a particular antigen. In light of these functions and our previous data demonstrating that rapamycin inhibited B cell class-switching, we hypothesized that rapamycin would lower *Pdk1* expression to enhance energy for antibody production and reduce assembly of high affinity influenza-specific antibodies.

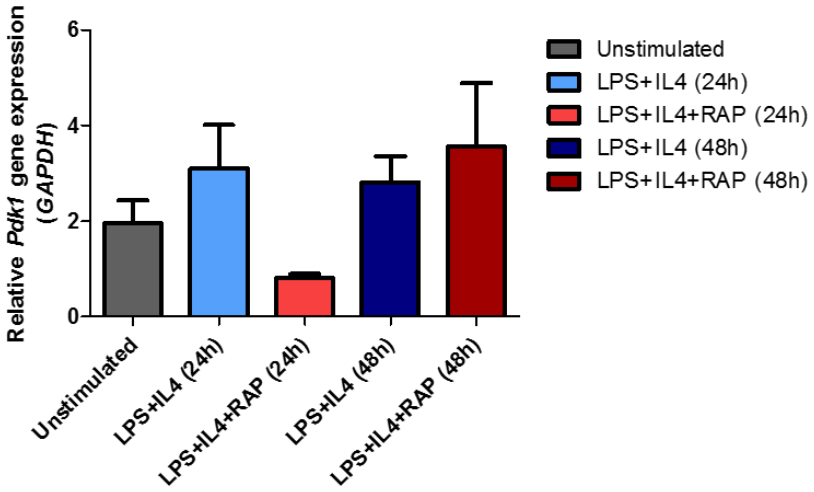


Figure 3-6: *Pdk1* Expression in Splenic B Cells

We found that B cells treated with rapamycin for 24 hours showed a decrease in Pdk1 mRNA relative to controls (**Figure 3-6**). However, this difference was not observed 48 hours after stimulation. These data suggest that rapamycin temporarily decreases *Pdk1* expression, which may impact the ability of the B cells to undergo class switching. However, the differences were not statistically significant, so further studies would be necessary to confirm this finding.

3.3b: *Slc2a1*

The *Slc2a1* (Solute Carrier Family 2 Member 1) gene encodes GLUT1 (Glucose Transporter Protein Type 1), which functions as the primary glucose influx transporter protein in B cells and various other cell types[62]. It has been shown that GLUT1 expression increases during B cell receptor stimulation to provide necessary fuel for maturation and proliferation[63] Thus, in the context of heterosubtypic protection, we hypothesized that rapamycin would decrease GLUT1 expression to reduce proliferation of B cells, thereby reducing the high affinity strain specific influenza antibodies, allowing the antibodies specific for the more conserved regions to be more prevalent.

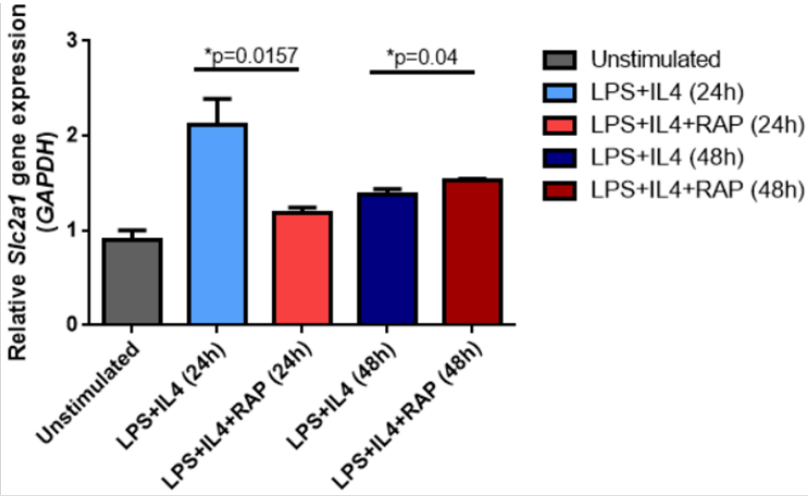


Figure 3-7: *Slc2a1* Expression in Splenic B Cells

To test whether the low dose of rapamycin impacted *Slc21a1* expression, we stimulated B cells as previously described. We found that *Slc2a1* expression significantly decreased with Rap treatment at 24 hours compared to controls, which

validates our hypothesis that Rap decreases glucose uptake by GLUT1 in stimulated B cells. This trend suggests that glucose regulation plays an important role in restricting the development of strain-specific antibodies, though more tests would be necessary to confirm a definitive connection between the two. Interestingly, the trends at 24h and 48h appear to differ in that rapamycin increased *Slc2a1* mRNA in 48h samples relative to controls. It should be noted, however, that this trend is consistent across every gene tested. Thus, it is not clear whether this difference is due to the stimulation conditions or if it is a true effect of rapamycin.

3.3c: *Xbp1*

The *Xbp1* (X-box Binding Protein 1) gene encodes a bZIP (basic Leucine Zipper domain) protein that binds a promoter region of MHC II, and controls the differentiation of B cells into antibody-secreting plasma cells[64,65]. Given that this process is essential for plasma cell differentiation, we tested whether the low dose of rapamycin would impact *Xbp1* expression[66].

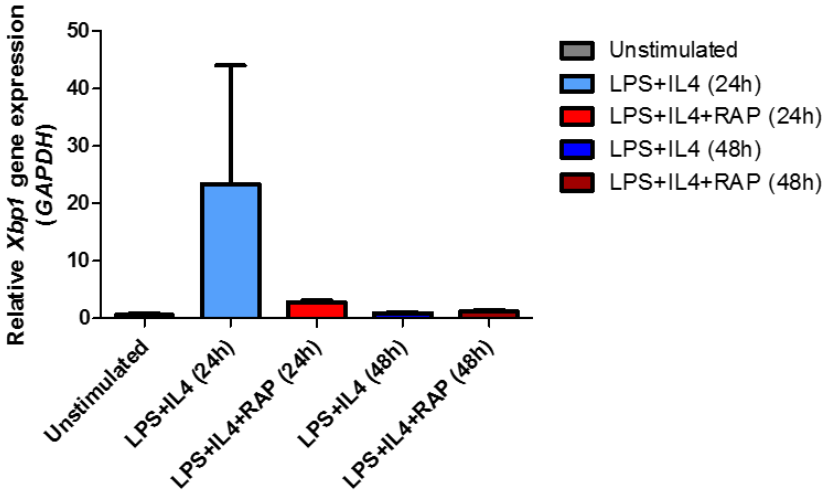


Figure 3-8: *Xbp1* Expression in Splenic B Cells

To test this, we tested B cells as previously described. Although there was variability between our technical replicates, our data suggest that rapamycin decreased *Xbp1* expression at 24 hours. By 48 hours Xbp1 expression was decreased in both groups. Therefore, these experiments will need to be repeated to determine if rapamycin does impact this pathway. If the trend at 24h holds up in future experiments, it might suggest that rapamycin restricts plasma cell differentiation to mitigate the response of strain-specific influenza antibodies.

3.3d: *Irf4*

Irf4 (Interferon Regulatory Factor 4) encodes a transcription factor that regulates interferons in response to viral infection[67]. In the context of heterosubtypic influenza protection, this gene is essential for germinal center formation in B cells, which is necessary for antibody class-switching and somatic hypermutation[47,66,68]. Based on the previous finding that rapamycin downregulates germinal center formation to partially

inhibit class-switching, we hypothesized that it might accomplish this through downregulation of *Irf4* expression[47].

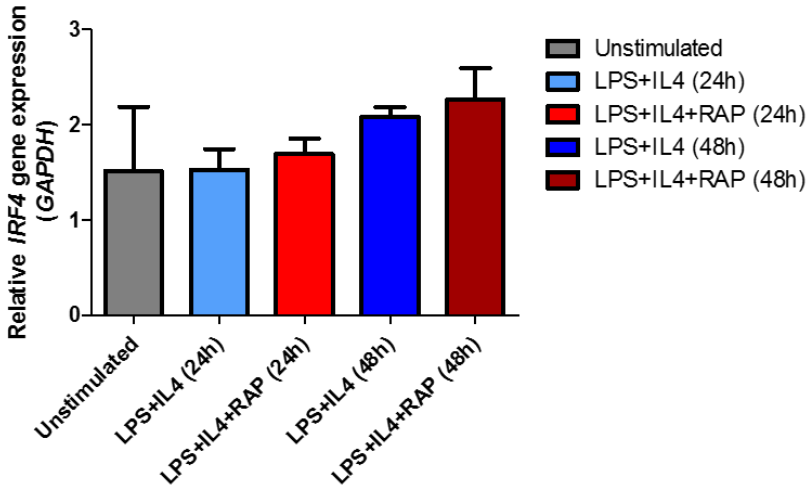


Figure 3-9: *Irf4* Expression in Splenic B Cells

We found that there were no significant differences in *Irf4* expression in cells treated with rapamycin or control. Thus, the low dose of rapamycin does not appear to regulate *Irf4* gene expression at these time points. Though this result is somewhat surprising, it may be rooted in *Irf4*'s dual role as a regulator of and requirement for plasma cell differentiation[68]. Since plasma cell differentiation is necessary for a robust antibody response, downregulation of this essential function through reduced *Irf4* could antagonize rapamycin's antibody-mediated protection. In addition, *Irf4* expression levels may be different in response to influenza versus LPS and IL-4.

3.3e: *Hif1α*

Hif1α (Hypoxia-Inducible Factor 1 alpha subunit) encodes the alpha subunit of the transcription factor HIF1, which affects a variety of pathways to maintain homeostasis in response to hypoxia[69]. One of *Hif1α*'s most prominent functions in B cells

is glycolysis regulation. During development, *Hif1α* ensures the efficient use of glucose by the B cell to maintain energy requirements at various stages of maturation[70]. Without *Hif1α*, B cells do not develop properly. It has been shown that mTORC1 activation induces HIF1α activity. Thus, we wanted to know whether low-dose rapamycin would block *Hif1α* which may impact heterosubtypic immunity. Based on its requirement for proper B cell development, we hypothesized that some downregulation might occur.

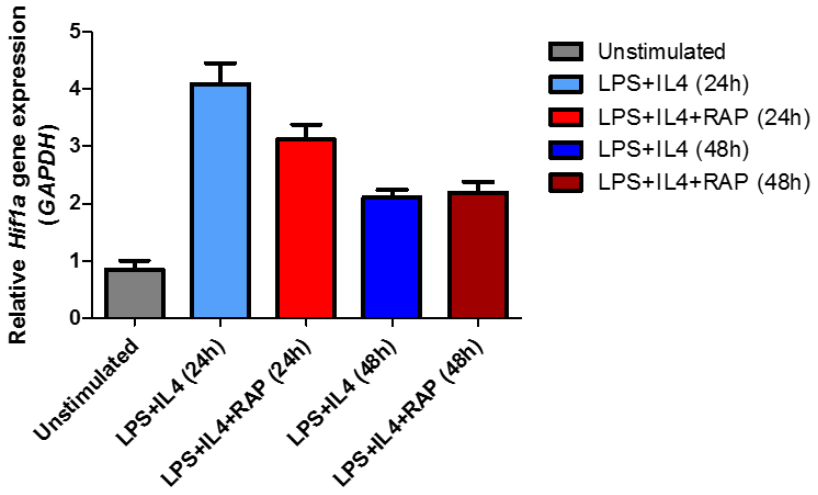


Figure 3-10: *Hif1α* Expression in Splenic B Cells

Despite a trend of lowered expression at 24h, *Hif1α* showed no significant differences in expression between stimulated control and rapamycin-treated B cells. Thus, we concluded that low-dose rapamycin does not significantly impact *Hif1α* gene expression.

3.3f: *c-Myc*

The *c-Myc* gene encodes a multifunctional, nuclear phosphoprotein transcription factor that regulates the cell cycle, apoptosis, and cellular transformation through

targeting of specific genes[71]. One of *c-Myc*'s many functions in B cells is the ability to regulate germinal center formation. Through analyzing expression of downstream *c-Myc* effectors, studies demonstrated the necessity of *c-Myc* expression for proper functioning of germinal centers[72]. Thus, we wanted to determine whether low-dose rapamycin altered the expression of *c-Myc* to mediate the assembly of conserved cross-reactive antibodies and inhibit class-switching. Based on its role in germinal center regulation, we hypothesized that *c-Myc* expression would be downregulated by low-dose rapamycin.

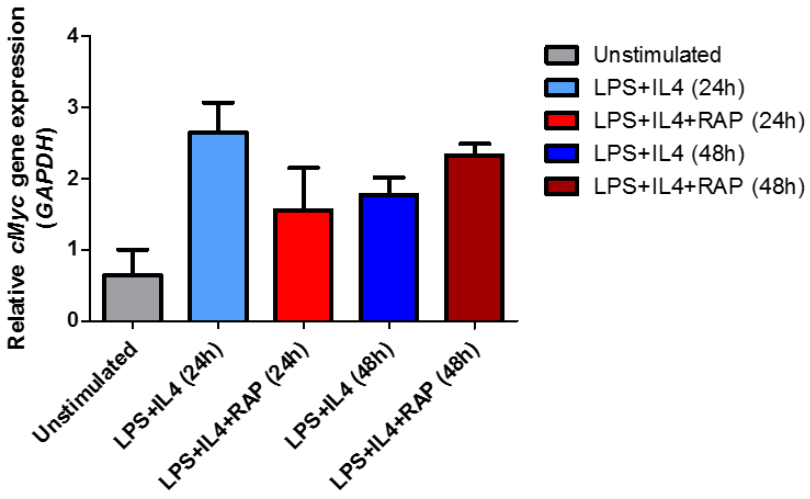


Figure 3-11: *cMYC* Expression in Splenic B Cells

Again, we found that there was a trend for decreased expression of *c-Myc* in the rapamycin-treated samples, but not a significant difference between the groups. If this trend was replicated in a repeat experiment with less variable data, it could indicate that rapamycin slightly decreases c-MYC expression, which may impact germinal center formation. Based on the current data alone, however, we must assume that low-dose rapamycin does not alter *c-Myc* expression to provide heterosubtypic influenza protection.

3.3g: *Bach2*:

The *Bach2* gene encodes a protein transcription factor that is required for class-switch recombination, somatic hypermutation, and germinal center formation in B cells[47,73,74]. Based on these functions, we hypothesized that low-dose rapamycin would lower *Bach2* expression to promote the reduction of these processes that we observed in previous experiments.

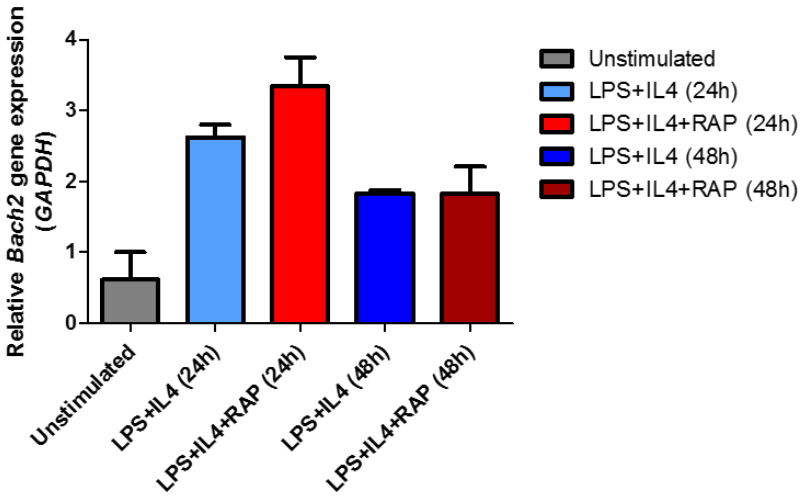
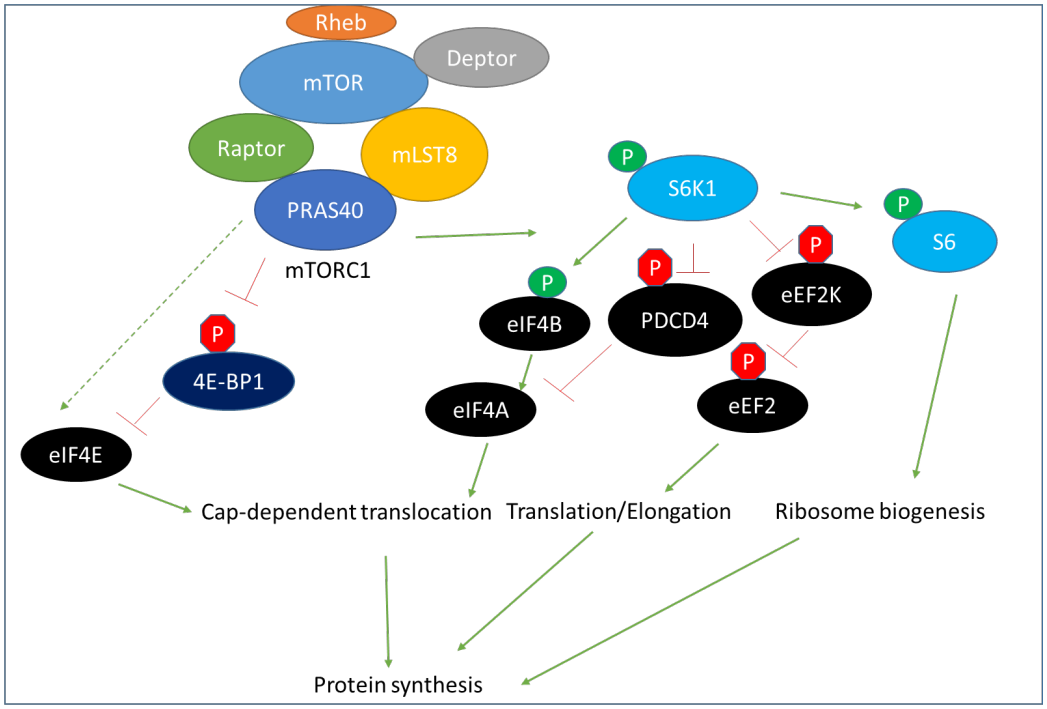


Figure 3-12: *Bach2* expression in splenic B cells

No significant differences were seen in *Bach2* expression during qPCR analysis of splenic B cells. Interestingly, the trend of increased expression shown in 24h Rap samples contradicts our assertion that rapamycin would decrease *Bach2* expression. If this trend persists in future experiments, it could mean that low-dose rapamycin uses *Bach2* regulation to help maintain levels of germinal center formation appropriate for the production of its conserved neutralizing antibodies. However, the current data indicate that rapamycin does not regulate *Bach2* expression to promote heterosubtypic protection.

3.4: THE ROLE OF LOW-DOSE RAPAMYCIN IN DOWNSTREAM PHOSPHOPROTEIN REGULATION



Supplemental Figure 3-1: mTORC1 Phosphorylation Signaling Pathway
Adapted from: 75. Laplante, M. & Sabatini, D. M. mTOR signaling at a glance. *J. Cell Sci.* 122, 3589–3594 (2009).

One of mTOR's best-characterized methods of cellular regulation is its ability to alter the phosphorylation state of downstream proteins upon activation. Given that rapamycin's basic mechanism of action is the inhibition of mTOR, it follows that some portion of its therapeutic effects must rely on its curtailment of these alterations. To discover which proteins might play a role in our low dose of rapamycin's provision of heterosubtypic influenza protection, we conducted western blots on a variety of known downstream mTORC1 proteins and compared their activation between control and rapamycin-treated B cells.

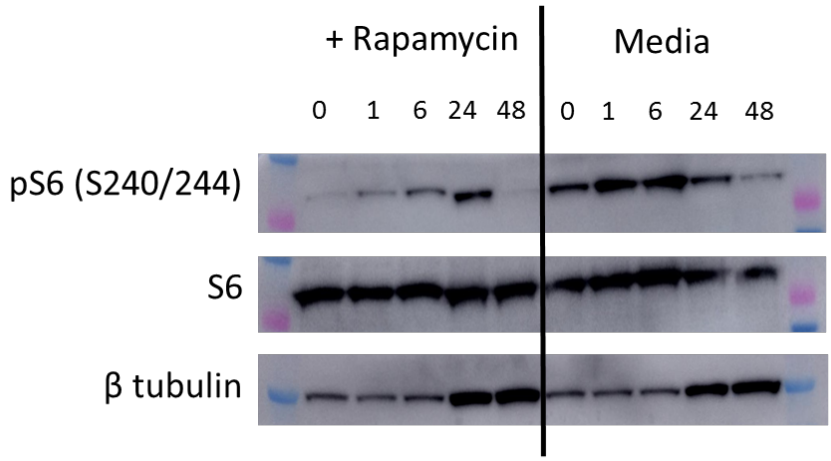


Figure 3-13: Expression of pS6, S6, and β tubulin in stimulated rapamycin and control B cells

The reduction in pS6 at earlier time points indicates that our low dose of rapamycin effectively decreases phosphorylation of S6 through its inhibition of mTOR (**Figure 3-13**). This result was validated using total S6 protein and β tubulin as loading controls to ensure differences in phosphorylation were not caused by disparities in protein quantity. Though it can be deduced from previous experiments, whose data are depicted above (**Supplemental Figure 3-1**), that this inhibition likely serves to reduce ribosome biogenesis, and thereby protein synthesis, it is unclear how these reductions might assist in the provision of heterosubtypic influenza protection.

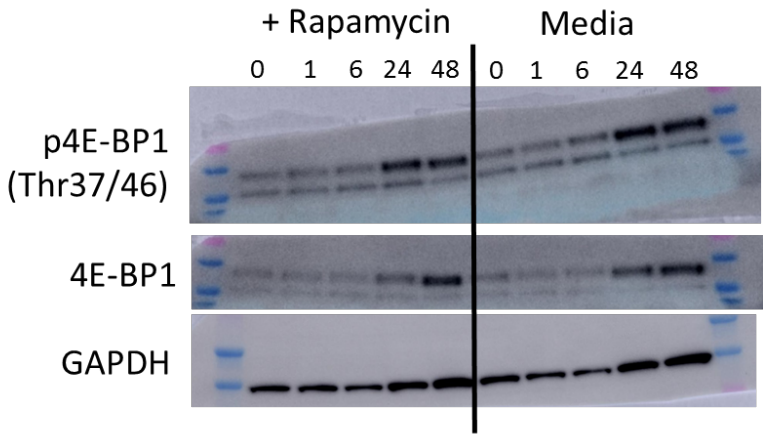


Figure 3-14: Expression of p4E-BP1, 4E-BP1, and GAPDH in stimulated rapamycin and control B cells

In response to stimulation, expression of p4E-BP1 steadily increased in both rapamycin-treated and control B cells, but was not impacted by the low dose of rapamycin (**Figure 3-15**). This result was validated by the use of total 4E-BP1 and GAPDH as loading controls to ensure comparable protein distribution across wells. Based on the data, and known functions of p4E-BP1, we conclude that low-dose rapamycin does not alter protein synthesis through regulation of cap-dependent translocation through this pathway.

3.5: LPS vs. anti-IgM STIMULATION COMPARISON

To determine if rapamycin may impact the mTOR pathway differently in response to other stimuli, we also analyzed the activation of S6 and 4EBP1 by flow cytometry following stimulation with anti-IgM and LPS.

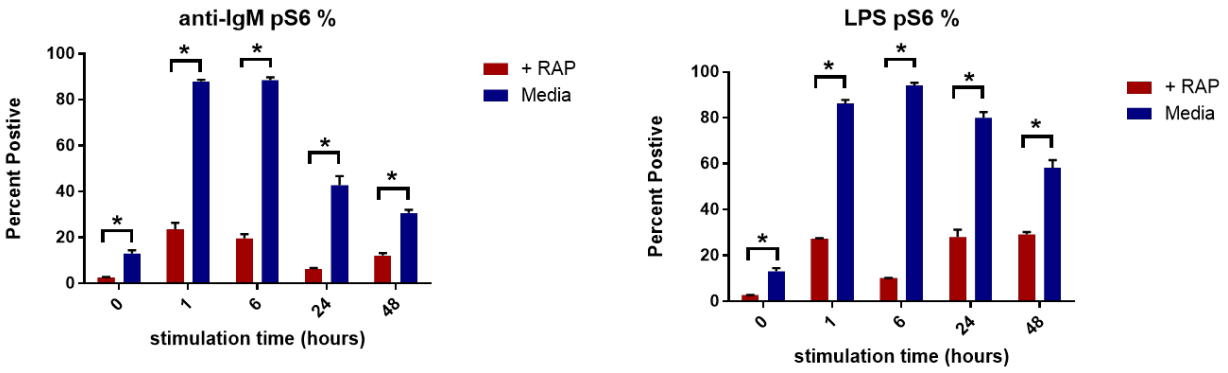


Figure 3-15: Percent Cells Positive for pS6 Expression in anti-IgM and LPS-stimulated B cells

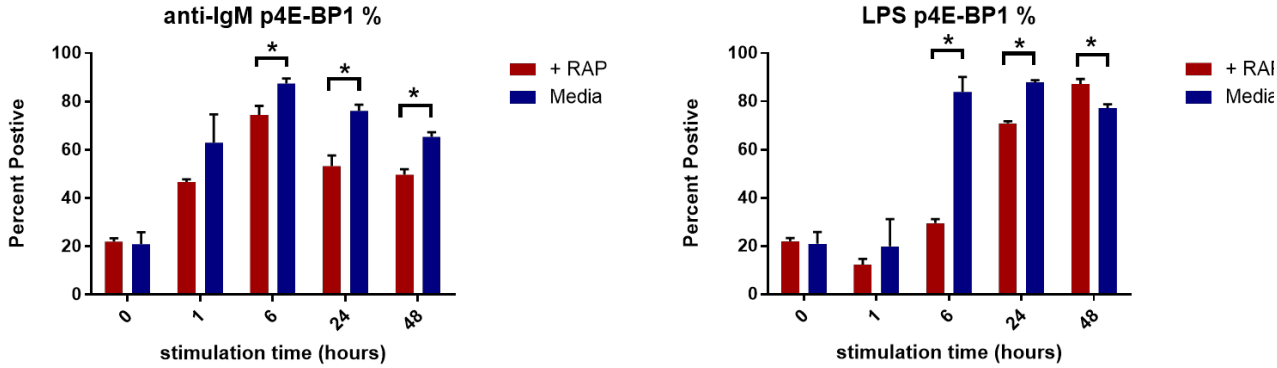


Figure 3-16: Percent Cells Positive for p4E-BP1 Expression in anti-IgM and LPS-stimulated cells

Although the stimuli do differ kinetically, the significant differences in phosphoprotein expressions between rapamycin-treated and control media cells are preserved across stimuli. In both cases, anti-IgM stimulated cells show an expression peak at 6 hours, before steadily decreasing as time goes on, while LPS-stimulated cells are more steadily expressed throughout stimulation. These trends indicate anti-IgM stimulation as a faster, more specific B cell stimulus than LPS. Since significant differences are consistent, however, the difference in these trends would not affect overall

expression data and interpretation. Thus, this experiment served to validate our previous results.

CHAPTER 4

DISCUSSION

4.1: THE FUNCTIONALITY OF RAPAMYCIN-MEDIATED HETEROSUBTYPIC INFLUENZA PROTECTION

A low dose of rapamycin (75 μg/kg) enhances protection against multiple subtypes of influenza when given during vaccination. However, the mechanisms of action remain unclear. Previous experiments showed that rapamycin promoted the generation of antibodies that did not neutralize influenza infection *in vitro.* Therefore, we tested other mechanisms by which non-neutralizing antibodies protect in order to further characterize the rapamycin-mediated response of these antibodies to influenza infection. One of the most prominent protective mechanisms of non-neutralizing antibodies is complement-mediated phagocytosis. This mechanism employs non-neutralizing antibodies to form antibody-antigen complexes in response to viral infection. These complexes expose antibody receptors for serum complement proteins to bind for the purpose of attracting macrophages with complement receptors that engulf and destroy virally-infected cells. We found that antibodies generated in the presence of rapamycin did not require activity of the complement system to provide protection. Depletion of complement *in vivo* did not significantly alter rapamycin-mediated protection following a lethal heterosubtypic infection. Rapamycin increased survival rates by 44% compared to PBS control mice when complement was present, and 57% in complement-depleted mice. These data suggest that low-dose rapamycin does not require complement to

enhance protection. If the complement system assisted rapamycin in providing heterosubtypic immunity, its depletion should have decreased rapamycin-mediated survival rates relative to controls. However, the opposite trend occurred here. While complement depletion did not significantly alter relative mortalities between control and CVF treatment groups, it did increase overall mortality in both PBS and rapamycin-treated mice, suggesting that the complement system plays an important role in the defense against influenza protection independent of rapamycin treatment.

Additionally, we found that antibodies generated in the presence of rapamycin did not exhibit increased levels of ADCC compared to antibodies generated in control mice. We tested antibodies from mice vaccinated with H3N2 and treated with rapamycin or a PBS control in an *in vitro* ADCC assay, and did not observe a difference in ADCC between the groups. These data do not rule out that ADCC is required for the antibodies to mediate protection, but there is not increased ADCC compared to antibodies in control mice. To further address whether ADCC was required, we depleted NK cells *in vivo* prior to challenge with the lethal heterosubtypic strain, and observed that NK cell depletion did not impact survival or weight loss rates *in vivo*, suggesting that ADCC is not required for rapamycin-mediated protection. While FcR binding did not significantly differ between antibodies of rapamycin-treated mice and antibodies of PBS-treated mice, both treatment groups did experience noticeable increases in binding after vaccination. These increases suggest that ADCC activity is present during the immune system's fight against influenza infection, and could play an important role in viral clearance of influenza, but it is not increased by rapamycin. Interestingly, the antibodies of rapamycin-treated mice appeared to bind FcRIV slightly more than the antibodies of PBS-treated mice. However, this

trend's extension to uninfected cells implies that the increases in binding may not be influenza-specific. Together, our data showed that ADCC activity was not increased in the serum of rapamycin-treated mice compared to control-treated mice after vaccination, and NK cells were not required for the rapamycin-mediated protection, suggesting that the antibodies generated in the presence of rapamycin do not utilize ADCC to mediate protection.

Given that ADCC and complement were not required for rapamycin to enhance heterosubtypic protection, it stands that another facet of the immune system must be employed to achieve heterosubtypic immunity. The most likely remaining mechanism of action for these antibodies is FcR-mediated phagocytosis. A previous study with an infection model similar to our own found that this mechanism was necessary for heterosubtypic influenza protection *in vivo*, but not *in vitro*[53]. This assertion would fit with our data on FcR binding since the lack of significant differences in binding between PBS and rapamycin treated mice was found *in vitro*. In the previous study, loss of protection was found with inhibition of Fcγ receptor action and depletion of alveolar macrophages through intranasal administration of clodronate-loaded liposomes[53]. Thus, this study provides a new, interesting lead in the hunt for the mechanism by which antibodies generated in the presence of rapamycin enhance protection against multiple subtypes of influenza.

4.2: REGULATION OF DOWNSTREAM mTOR EFFECTORS BY LOW-DOSE RAPAMYCIN

In order to understand how a low dose of rapamycin enhances heterosubtypic immunity, we analyzed its impact on genes known to be downstream of mTOR. Through qPCR analysis, we found that a low dose of rapamycin reduced expression of *Slc2a1* in B cells following *in vitro* stimulation. The downregulation of *Slc2a1* reduces cellular glucose uptake by partially inhibiting transcription of primary glucose transporter protein GLUT1. While a definitive relationship between glucose regulation and heterosubtypic protection has not yet been established, its existence seems likely based on previous research. Studies showed that B cells increase glucose uptake via GLUT1 in the pro-B cell stage, and in response to anti-IgM stimulation[70,63]. These findings suggest that B cells have higher energy requirements at these times.

Both the pro-B cell stage and IgM stimulation are critical for generating specific antibodies. During the pro-B cell stage of development, antibody heavy chains undergo V-DJ recombinations that alter the specificity of the antibody heavy chain, which impacts what antigens the antibody binds, and is required for further development and proliferation of B cells[35]. Thus, by downregulating *Slc2a1* expression in pro-B cells, a low dose of rapamycin may restrict glucose intake enough to reduce energy levels beneath the threshold required to maintain these recombinations. In this way, rapamycin could inhibit the production of new B cells that have strain-specific antibodies. The reduction of *Slc2a1* expression by rapamycin may also limit antibody specificities after V-DJ recombination by inhibiting germinal center activity. Since glucose intake increases during germinal center formation, which occurs in response to antibody stimulation,

limiting glucose intake would limit energy for class switch recombination and somatic hypermutation. Both class switch recombination and somatic hypermutation in the germinal centers are the main mechanisms by which high affinity antibodies that are specific for the variable regions of influenza HA develop. In the germinal centers, the antibodies with the highest affinity for the pathogen are selected to develop, and outcompete other antibodies of lower affinities[33]. For influenza, the highest affinity antibodies typically bind to the globular head of the HA protein, which in turn, causes the globular head to be the most variable region of the virus. We hypothesize that a low dose of rapamycin partially blocks the germinal center formation and generation of the high affinity antibodies, which allows the lower affinity antibodies to the conserved portions of the virus to be more prevalent. It is possible that reduction of *Slc2a1* may mediate this block in germinal centers, but, further tests would have to be conducted to validate this hypothesis.

Interestingly, the trend of rapamycin-mediated *Slc2a1* regulation seemed to switch from downregulation at 24 hours to upregulation at 48 hours. Although the trend of upregulation is significant, its validity is questionable for two reasons. First, the trend of increased genetic expression in rapamycin-treated cells relative to controls was present for every gene tested at 48 hours. While it is possible that rapamycin actually increased expression for all 7 genes tested at 48 hours, it is more probable that this trend stems from the delayed kinetics of LPS stimulation. As shown in Figures 3-16 and 3-17, LPS stimulates B cells at slower rate than anti-IgM. As a result, gene expression in LPS and rapamycin-treated cells may appear higher at 48 hours because it is delayed compared to IgM stimulation. Thus, a repeat of these experiments with anti-IgM stimulation would be

valuable to confirm conclusions and nonsignificant trends in expression. Additionally, the margin of difference in relative *Slc2a1* expression between stimulated controls and stimulated rapamycin-treated cells drops 2.5 percentage points from the 24 hours to 48 hours. Thus, the significance of the difference at 48 hours is diminished, casting further doubt on its validity.

Another potential flaw in the qPCR results was large RQ (relative quantity of gene expression) variability within treatment groups. The most glaring instance of this trend occurred during *Xbp1* analysis (**Figure 3-9**). While most RQ values range between 0 and 3, *Xbp1* showed RQ values upward of 500 in both stimulated controls and rapamycin-treated cells at 24 hours. After excluding these values, a 24 hour stimulated control sample with an RQ of approximately 64 remained, which accounts for its exaggerated expression and error bar relative to other *Xbp1* treatment groups. Similar variabilities occurred to lesser extents with other genes. These variabilities may have resulted from technical errors and deficiencies in eluted RNA quantity. For this reason, and the potential effects of LPS stimulation, these experiments will need to be repeated to confirm any nonsignificant trends seen in our data.

We also found that rapamycin reduced pS6 expression *in vitro* via western blot analysis. During activation, mTORC1 indirectly phosphorylates pS6 to promote ribosome biogenesis for protein synthesis[75]. Thus, a low dose of rapamycin inhibited mTOR sufficiently to prevent phosphorylation of pS6. Use of total protein controls S6 and β tubulin confirmed that differences in expression were not due to sample loading errors. While it is unclear how rapamycin-mediated inhibition of ribosome biogenesis, and subsequent protein synthesis, might directly promote heterosubtypic influenza protection,

it is interesting to note that this reduction only occurs at early time points of 0, 1, and 6 hours. Since we controlled for the half-life of rapamycin in 48 hour samples by providing an extra dose of rapamycin (1 ng/well) and did not observe any further alteration of pS6 expression, these trends may indicate that mTOR exhibits a decreased sensitivity to rapamycin after only initial stimulation *in vitro*.

Overall, our data indicate that low-dose rapamycin does not require the activity of the complement system or ADCC to provide optimal heterosubtypic immunity, but may promote heterosubtypic protection by reducing the expression of *Slc2a1* and pS6. Although the protective mechanisms of rapamycin-mediated non-neutralizing antibodies remain unclear, the elimination of the complement system and ADCC from the pool of suspects leaves us one step closer to finding the true mechanism by which these antibodies function. The reduction of *Slc2a1* expression by low-dose rapamycin confirms that rapamycin influences genetic regulation, although the specific mechanisms remains unclear. Finally, the inhibition of pS6 phosphorylation, coupled with the lack of activity on p4E-BP1, indicates the partial block of mTOR by the low dose of rapamycin is specific in its alterations of downstream effectors. Understanding how rapamycin alters the immune system to enhance immunity to multiple subtypes of influenza will aid in developing a universal influenza vaccine, which could potentially save millions of lives in the event of a deadly pandemic.

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